Effect of nanoparticle conjugation on siRNA gene silencing

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Materials. Quantum dots with emission maxima of 655 nm and modified with PEG and amino groups were obtained from invitrogen. QD concentrations were measured by optical absorbance at 490 nm, using extinction coefficients provided by the supplier. Cross-linkers used were sulfosuccinimidyl 6-(3-[2-pyridyldithio]- propionamido)hexanoate (SPDP), sulfosuccinimidyl 6-[α-methyl-α-(2-pyridyldithio)toluamido] hexanoate (SMPT) and non-labile thioether forming Succinimidyl-[(N-maleimidopropionamido)-$_n$ethylene glycol] ester (NHS-PEO$_n$-Maleimide) with n=4,12 and 24. All the crosslinkers were purchased from Pierce. Synthetic RNA duplexes directed against Luciferase and CXCR4 gene were synthesized (Alnylam), with the sense strand or the antisense strand modified to contain a 3' thiol group. The sequence for Luc siRNA used is 5'-GCCAAGAAGUUUCCUAAUAdTdT (sense strand) and 5'-UAUUAGGAAACUUCUUGGCdTdT (antisense strand). The sequence for CXCR4 siRNA used is 5'-GUUUUCACUCCAGCUAACAdTdT-3' (sense strand) and 5'-UGUUAGCUGGAGUGAAAACdTdT-3' (antisense strand).

Conjugation of siRNAs to QDs:

Amino-modified QDs were conjugated to thiol-containing siRNA using SPDP, SMPT and NHS-PEO$_n$-Maleimide (n= 4, 12 and 24) cross-linkers. QDs were resuspended in RNAse free 50 mM sodium phosphate, 150 mM sodium chloride, pH 7.2, using Saphadex G-25 gravity column (NAP-5, GE Healthcare). Cross-linker (1000-fold excess) was added to QDs and allowed to react for 2 h. Samples were filtered on a NAP-5 column (to remove excess cross-linker) into similar buffer supplemented with 10 mM EDTA. siRNA was treated with 0.5 M DTT for 1 h and filtered on a NAP-5 column into EDTA-containing buffer. A 10-fold molar excess of siRNA was

added to the filtered QDs and allowed to react overnight at 4 °C. Using Amicon filters, product was filtered twice with Dulbecco's phosphate buffered saline (PBS), twice with a high salt buffer (1.0 M sodium chloride, 100 mM sodium citrate, pH 7.2) required to remove electrostatically bound siRNA. The samples were filtered several times (~12) with PBS until the supernatant showed no free siRNA by UV-Vis spectroscopy (Figure S1a). The conjugation of siRNA to the QD was confirmed and quantified using gel electrophoresis and Fluorescence spectroscopy. The samples were incubated with 10 mM Glutathione for 2h at 37 °C and run on a precast 15% TBE gel (BioRad Laboratories) at 80 mV for 1 h. The siRNAs in the gel were stained with SYBR Gold (Invitrogen Inc., Carlsbad, CA). To quantify the number of siRNA on QD, the samples were run along with free siRNA standards on the gel. The number of siRNA was found to be similar for both the labile (SPDP and SMPT) cross-linkers (Figure S1b). Since, the siRNA on the QDs with non-labile cross-linkers cannot be reduced by glutathione, to confirm and quantify the amount of siRNA on the QD with NHS-PEOn-Mal cross-linkers were stained with SYBR Gold and measured with a fluorimeter (SpectraMax Gemini XS, Molecular Devices). Similar loading of siRNA was observed for both non-labile (Mal-12 and Mal-24) cross-linkers (Figure S1c). To assess the purity of the QD-siRNA conjugates with non-labile cross-linkers knockdown experiment (Figure S2) was performed with the filtrate obtained after the tenth centrifugation cycle of the non-labile samples.

Cell Culture:

The knockdown experiments were performed on HeLa(EGFP-Ago2) cell line generated as described previously. Growth media was Dulbecco's modified Eagle's medium (DMEM)

containing 4.5 g/L glucose and supplemented with 10% FBS, 500 ng/mL puromycin and 600 μg/ml G418 for selection. One day before transfection, cells were seeded at 5 x 10^5 cells/well in a 6-well plate such that they would be 95% confluent at the time of transfection. For transfection, QD-siRNA conjugates, free siRNA (5 μM) and non-conjugated QD were electroporated by using I-13 protocol (Amaxa, Gaithersburg, MD). As transfection efficiency is affected by the concentration of siRNA, we optimized the concentration of free siRNA that gives the most efficient transfection by this method of delivery (electroporation). The dose-dependent relationship between the Luciferase gene silencing and the siRNA concentration can be seen in Figure S3. A final QD concentration of 20 nM was used per well for each sample. The medium was changed 4 h post transfection, and cells were incubated for another 44 h before they were washed with PBS, lysed with passive lysis buffer (Promega), and assayed for luciferase expression with a Dual-Glo luciferase assay kit (Promega) on a single tube luminometer (GloMax, Promega). The cell lysates were also used for measuring the total protein concentration for each sample using the Bradford reagent (Bio-Rad, Hercules, CA, USA). Since the efficiencies of the free siRNA (5 μM) and 20 nM QD-MAL-siRNA (60-80 nM siRNA concentration) are not directly comparable six different concentrations of free CXCR4 siRNA and 60-80 nM of the QD conjugated siRNA (S-QD-4-CXCR4 and S-QD-24-CXCR4) were electroporated in the modified Hela cells. Assessment of the luciferase activity after 48 h of transfection revealed that the knockdown due to the QD-conjugated siRNA was similar to the knockdown obtained by free siRNA at the concentration of 100 nM. The results indicate that the conjugates (S-QD-24-CXCR4) have comparable or marginally higher silencing efficiency compared to the free siRNA at similar concentrations.

Stability of QD-siRNA conjugates in serum:

The QD-siRNA conjugates (20 nM) were incubated in 10% FBS for specified amount of time at 37 °C. The samples were then centrifuged using Amicon filters (MWCO 30,000) twice to remove all the free siRNA (not remaining on the NP) from the sample after serum exposure. The clean samples were then used for visualizing the siRNA remaining on the QD and to perform knockdown experiments with the conjugates after serum treatment. For visualizing the siRNA remaining on the QD (after serum treatment), the filtered samples were further incubated with 10 μM glutathione for 2h at 37 °C and analyzed by gel electrophoresis (15% TBE gel) and SYBR gold staining as described above. The knockdown experiments with filtered-serum-treated QDs were performed using a similar protocol as described above.

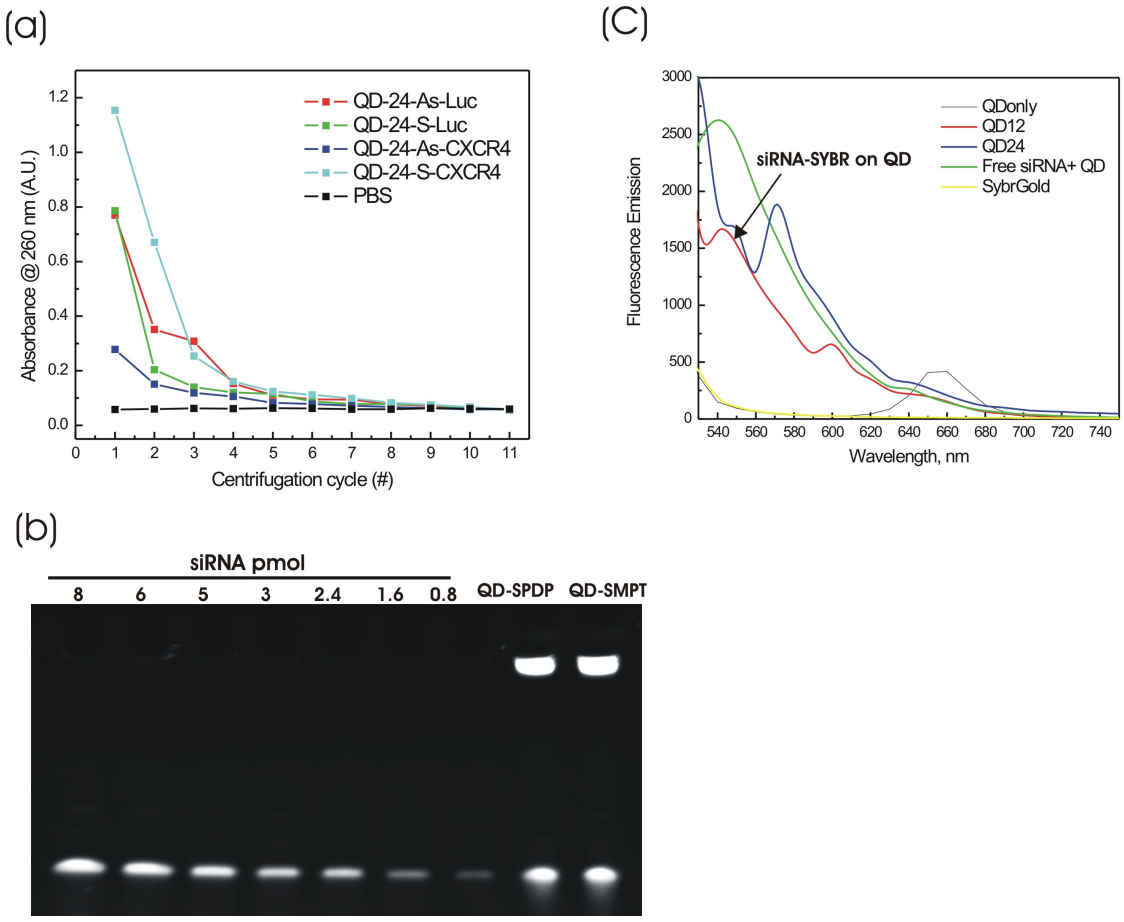

Figure S1. (a) Purification of QD-siRNA conjugate by centrifugation. The removal of free siRNA was monitored in the sample by measuring the absorbance of the filterate after each cycle at 260 nm. (b) Amount of siRNA conjugated to the QDs using labile cross-linkers. (c) Presence of siRNA on QD-siRNA conjugates with non-labile cross-linkers.

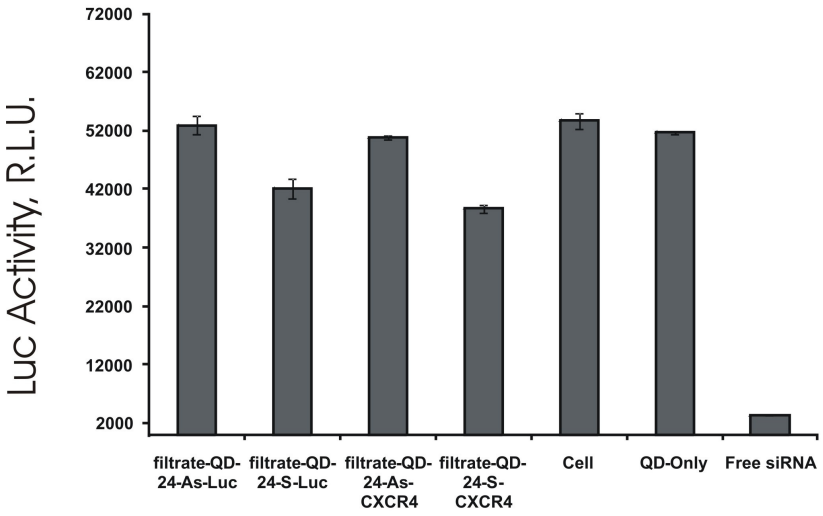

Figure S2. Luciferase knockdown by the filtrate obtained from the non-labile sample purification cycle. This demonstrates that the filtration process results in highly pure siRNA conjugated nanoparticles and there is no free siRNAs in the nanoparticle solution, hence the KD obtained by the nanoparticles is due to the conjugated siRNA.

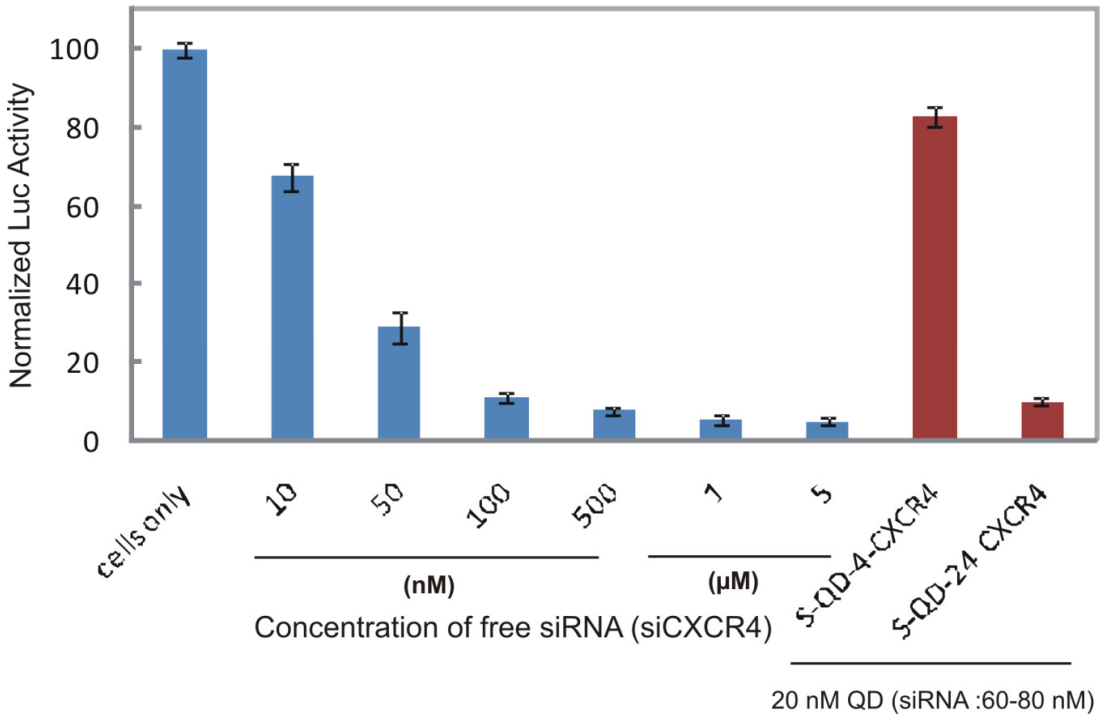

Figure S3. Luciferase knockdown by various concentrations of free siCXCR4 and 20 nM S-QD-4-CXCR4 (siRNA concentration of 60-80 nM). This indicates that the conjugates (S-QD-24-CXCR4) have comparable or marginally higher silencing efficiency compared to the free siRNA at similar concentrations.